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Expression, refolding and crystallizations of the Grb2-like (GADS) C-terminal SH3 domain complexed with a SLP-76 motif peptide

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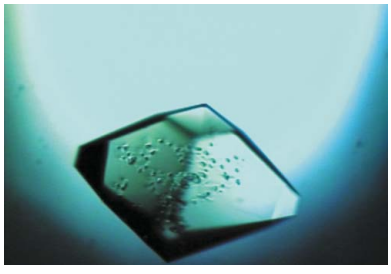


The Grb2-like adaptor protein GADS is composed of an N-terminal SH3 domain, an SH2 domain, a proline-rich region and a C-terminal SH3 domain. GADS interacts through its C-terminal SH3 domain with the adaptor protein SLP-76, thus recruiting this protein and other associated molecules to the linker for activation of T-cell (LAT) protein. The DNA encoding the C-terminal SH3 domain of GADS (GADS-cSH3) was assembled synthetically using a recursive PCR technique and the protein was overexpressed in *Escherichia coli*, refolded and purified. Several crystals of this domain in complex with the SLP-76 peptide were obtained and characterized.

1. Introduction

The identification of a family of proteins commonly known as adapters or molecular scaffolds has been a major advance in understanding T-lymphocyte signalling (Jordan *et al.*, 2003). By definition, these proteins lack any enzymatic activity or any transcription activation domains. Instead, they possess binding sites or distinct modules that bind to other proteins. Members of the adapter family in the T lymphocytes include LAT (linker for activation of T cell), SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa), VAV (guanine nucleotide-exchange factors of the Rho family), GADS (Grb2-related adapter downstream of Shc), ADAP (adhesion- and degranulation-promoting adapter protein) and SKAP55 (Src-kinase-associated protein of 55 kDa) (Samelson, 2002).

These adapter proteins include SH2 and PTB domains (which bind to phosphotyrosine motifs), SH3 domains (which bind to polyproline II motifs), PH domains (which interact with phospholipids), PDZ domains (which bind to hydrophobic motifs) and WW domains (which bind to proline-rich regions and phosphoserine residues). With such an architecture, a single modular protein can participate in and regulate several discrete intracellular events and/or provide a scaffold for grouping other proteins into complexes in signal transduction pathways.

Src homology 3 (SH3) domains were the first recognition modules to be identified (Musacchio, 2002). Generally, SH3 domains recognize specific proline-rich sequences that are found in both prokaryotic and eukaryotic proteins. These domains are conserved from yeast to mammals, suggesting that they could play a pivotal role in the higher eukaryotes. The SH3 domain is generally short and compact, comprising about 50–70 amino-acid residues. To date, the three-dimensional structure of more than 130 proteins containing SH3 domains, either non-complexed or in complex with their respective ligands, have been determined. The prototype SH3-domain structure (Musacchio, 2002) is composed of five β-strands (β_A–β_E) and a single turn of 3_{10}-helix. The five β-strands are arranged to form two sheets.

GADS is an intracellular signalling protein composed of an SH3 domain and an SH2 domain at the N-terminus and a C-terminal SH3 domain interconnected by a long unstructured amino-acid sequence (Liu & McGlade, 1998). GADS binds several intracellular signalling proteins including SLP-76, LAT, c-Cbl, HPK1 and Gab3. SLP-76 possesses several tyrosine-phosphorylation sequences at the N-terminus, a central proline-rich region and a C-terminal SH2 domain. The C-terminal SH3 domain of GADS binds an SLP-76 proline-rich region. This region has been precisely mapped and surprisingly is

almost identical to the SLP-76 region which binds the C-terminal SH3 domain of Grb2 (Berry *et al.*, 2002; Liu *et al.*, 2001; Lewitzky *et al.*, 2001, 2004; Yoder *et al.*, 2001). Solution and structural studies of the C-terminal GADS SH3 domain in complex with the atypical SLP-76 consensus motif (Liu *et al.*, 2003; Harkiolaki *et al.*, 2003; Faravelli & Dimasi, 2006) and HPK1 (haematopoietic progenitor kinase 1; Lewitzky *et al.*, 2004) have been reported, revealing a new mode of binding interaction within the SH3 domains.

As a first step in dissecting the structural features of the modular proteins involved in T-cell receptor signal transduction pathways, we describe preliminary efforts into the crystallization of the C-terminal domain of the adapter molecule GADS complexed with the minimum SLP-76 binding motif.

2. Materials and methods

2.1. Expression and purification

The synthetic gene construction and recombinant expression of the carboxy-terminal SH3 domain of GADS (identified in this paper as

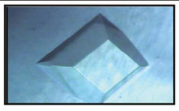
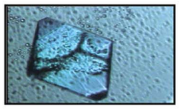
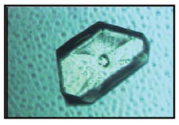
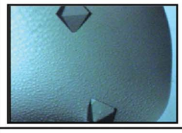
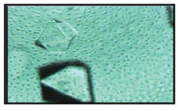
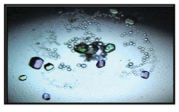
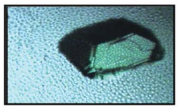
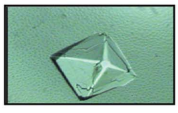
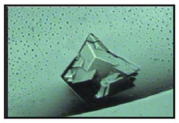
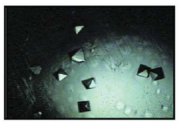
Crystal name	Crystal picture	Crystallization buffer	Crystal unit-cell parameters and space group	X-ray diffraction (λ = 1.431 Å) and data-collection statistics	
wi13		1.26 *M* $(NH_4)_2SO_4$ 0.1 *M* cacodylate pH 6.5	$a = b = 58.62$, $c = 74.09$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 45.97–1.78 Å Total reflections: 67350 Unique reflections: 12895	Completeness (%): 99.7 (100) R_{sym} (%): 8.5 (29.3) $\langle I/\sigma(I)\rangle$: 9.3 (3.2)
wi18		1 *M* Na tartrate 0.1 *M* imidazole pH 8.0 0.2 *M* NaCl	$a = b = 58.45$, $c = 75.85$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 46.30–1.94 Å Total reflections: 66890 Unique reflections: 13123	Completeness (%): 99.5 (98) R_{sym} (%): 4.0 (22.3) $\langle I/\sigma(I)\rangle$: 12.2 (4.3)
wi37		2.5 *M* NaCl 0.1 *M* imidazole pH 8.0	$a = b = 58.49$, $c = 75.83$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 46.31–1.94 Å Total reflections: 68870 Unique reflections: 12858	Completeness (%): 98.5 (100) R_{sym} (%): 7.2 (22.4) $\langle I/\sigma(I)\rangle$: 8.4 (3.8)
wi38		1.0 *M* Na tartrate 0.1 *M* CHES pH 9.5 0.2 *M* Li_2SO_4	$a = b = 58.43$, $c = 76.48$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 58.44–1.75 Å Total reflections: 67458 Unique reflections: 13132	Completeness (%): 99.5 (96) R_{sym} (%): 6.5 (23.4) $\langle I/\sigma(I)\rangle$: 10.4 (4.4)
wii37		1.0 *M* Na tartrate 0.1 *M* Tris pH 7.0 0.2 *M* Li_2SO_4	$a = b = 58.53$, $c = 75.17$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 46.18–2.02 Å Total reflections: 65587 Unique reflections: 12568	Completeness (%): 96.5 (100) R_{sym} (%): 7.5 (26.8) $\langle I/\sigma(I)\rangle$: 16.5 (3.2)
wi47		1.26 *M* $(NH_4)_2SO_4$ 0.1 *M* Tris pH 8.5 0.2 *M* Li_2SO_4	$a = b = 58.23$, $c = 75.78$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 46.65–1.53 Å Total reflections: 68756 Unique reflections: 13324	Completeness (%): 98.5 (100) R_{sym} (%): 8.0 (28.4) $\langle I/\sigma(I)\rangle$: 7.4 (3.1)
wii15		1.26 *M* $(NH_4)_2SO_4$ 0.1 *M* HEPES pH7.5	$a = b = 58.42$, $c = 75.15$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 54.44–1.98 Å Total reflections: 66658 Unique reflections: 14545	Completeness (%): 89.5 (95) R_{sym} (%): 3.8 (32.5) $\langle I/\sigma(I)\rangle$: 8.3 (3.1)
wii29		1.26 *M* $(NH_4)_2SO_4$ 0.1 *M* CHES pH 9.5 0.2 *M* NaCl	$a = b = 58.25$, $c = 74.58$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 38.45–2.05 Å Total reflections: 66987 Unique reflections: 12758	Completeness (%): 95.5 (89) R_{sym} (%): 4.5 (30.3) $\langle I/\sigma(I)\rangle$: 11.5 (3.8)
wii31		1.0 *M* sodium citrate 0.1 *M* Tris pH 7.0 0.2 *M* NaCl	$a = b = 58.76$, $c = 75.48$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 34.78–1.85 Å Total reflections: 67850 Unique reflections: 13132	Completeness (%): 99.5 (100) R_{sym} (%): 8.2 (26.5) $\langle I/\sigma(I)\rangle$: 18.5 (5.4)
wii16		1.0 *M* sodium citrate 0.1 *M* CHES pH 9.5	$a = b = 58.82$, $c = 75.74$ Å $\alpha = \beta = \gamma = 90°$ $P4_22_12$	Resolution: 43.64–1.74 Å Total reflections: 67853 Unique reflections: 12458	Completeness (%): 98.5 (100) R_{sym} (%): 4.5 (23.6) $\langle I/\sigma(I)\rangle$: 9.6 (3.3)

Figure 1
Crystals and X-ray data-collection statistics of the C-terminal SH3 domain of GADS in complex with the SLP-76 peptide. Values in parentheses correspond to the highest resolution shell. $R_{sym} = \sum |I_j - \langle I\rangle| / \sum I_j$, where I_j is the intensity of an individual reflection and $\langle I\rangle$ is the average intensity of that reflection.

GADS-cSH3, and corresponding to amino acids 267–322 of the GADS protein; NCBI GenBank entry O89100) are described elsewhere (Faravelli & Dimasi, 2006). Here, a short summary of the *in vitro* gene assembly, protein expression and purification is presented. A mixture of 14 chemically synthesized oligonucleotides, carrying optimized DNA codons for maximum protein expression in *Escherichia coli*, was used in order to assemble the DNA coding sequence for the carboxy-terminal SH3 domain of GADS. This DNA sequence was assembled using a recursive PCR technique as described previously (Dimasi *et al.*, 2002). The synthetic gene was designed to facilitate cloning into a pT7-based expression vector cleaved with *Nde*I and *Bam*HI restriction enzymes. The plasmid carrying the GADS-cSH3 domain was transformed into *E. coli* BL21 Rosetta strain (Stratagene, USA) and protein expression was achieved by adding 1 m*M* isopropyl β-D-thiogalactoside for 3 h at 310 K. Under these conditions, the GADS-cSH3 domain was expressed as inclusion bodies at about 50 mg per litre of bacterial culture. The cells were resuspended in 100 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA, 1 mg ml^{-1} lysozyme and lysed by sonication using Soniprep500 (Sanyo, USA). The inclusion-body pellet was washed several times in a buffer containing 50 m*M* Tris–HCl pH 8.0, 0.5%(*v*/*v*) Triton X-100, 100 m*M* NaCl, 1 m*M* EDTA, 10 m*M* DTT and solubilized in 100 m*M* Tris–HCl pH 8.0, 8 *M* urea, 10 m*M* EDTA, 10 m*M* DTT. Urea-solubilized GADS-cSH3 domain was folded *in vitro* at 277 K by slow dilution into 1 l of 0.8 *M* arginine, 100 m*M* Tris–HCl pH 8.0, 10 m*M* EDTA, 10 m*M* DTT with or without 1 mg ml^{-1} of the SLP-76 peptide of sequence H_2N-PSIDRSTKP-COOH corresponding to amino acids 233–241 of the entire SLP-76 protein (NCBI GenBank entry AAC50135). The refolded GADS-cSH3–SLP-76 complex and the GADS-cSH3 domain were purified by size-exclusion chromatography using a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech, USA) equilibrated in 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl at 293 K. The GADS-cSH3–SLP-76 complex elutes from the gel-filtration column as a *bona fide* dimer. In contrast, GADS-cSH3 uncomplexed with the SLP-76 peptide elutes under the same experimental condition as a *bona fide* monomer (Faravelli & Dimasi, 2006).

2.2. Crystallization and X-ray data collection

Crystallization trials of the GADS-cSH3–SLP-76 complex and GADS-cSH3 were performed by the hanging-drop vapour-diffusion method (McPherson, 1982) using a 24-well VDX plate (Hampton Research, USA) at 293 K. Crystallization drops were prepared by mixing 2 µl GADs-cSH3–SLP-76 or GADS-cSH3 with the same volume of reservoir solution. The drops were placed over 0.6 ml reservoir solution. The initial search for crystallization conditions was performed using commercially available kits from Hampton Research, Jena Bioscience and Emerald Biostructures and in-house crystallization screening kits. Of about 850 condition screened, several crystals were obtained after 1–3 d (Fig. 1). These crystals were well shaped, reproducible and of good diffraction quality (Fig. 1). Interestingly, crystals as shown in Fig. 1 were only obtained using protein solution containing the GADS-cSH3–SLP-76 complex. No crystals were obtained when the protein solution contained GADS-cSH3 uncomplexed with the SLP-76 peptide. SDS–PAGE and MALDI–TOF analysis of dissolved crystals confirmed the presence of both GADS-cSH3 and SLP-76 peptide (data not shown).

2.3. Data collection

All the crystals shown in Fig. 1 were used for data collection. These crystals mounted in cryoloops were dipped into cryoprotectant solution (reservoir solution supplemented with 15% glycerol) for a few seconds and immediately transferred into liquid nitrogen. The crystals were stored in liquid nitrogen and transferred to a nitrogen stream at 100 K before data collection. X-ray diffraction data were measured from the crystals listed in Fig. 1 on beamline X8C of the Brookhaven National Synchrotron Light Source using a Quantum 4 CCD detector (Area Detector Systems, USA). The raw data were processed and scaled using the program *HKL*2000 (Otwinowski & Minor, 1997; HKL Research Inc., USA). Summaries of crystallization and crystallographic data for all crystals used are shown in Fig. 1.

3. Results

Complete data sets for all the crystals shown in Fig. 1 were collected to high resolution on a synchrotron source. Their processing statistics are reported in Fig. 1. Even though all crystals were obtained using different buffer compositions, they all belong to the primitive tetragonal space group, with very similar unit-cell parameters. This was unexpected because different crystallization conditions for the same protein or same protein complex often result in different crystal forms and parameters. A Matthews analysis (Matthews, 1968) revealed that the most probable content of the crystals' asymmetric unit is a molecule with a molecular weight of approximately 15.5 kDa, with a Matthews coefficient of about 2.08 Å^3 Da^{-1} and a solvent content of about 41%. This analysis suggests that the crystal asymmetric unit could be composed of a GADS-cSH3–SLP-76 homodimer.

The structure for crystal wi13 was determined by molecular replacement (Fig. 2) using the program *PHASER* (Storoni *et al.*, 2004) and the coordinates of a previously determined GADS carboxy-terminal SH3 domain (PDB code 1oeb) as a search model. A preliminary analysis of this complex structure revealed, in agreement

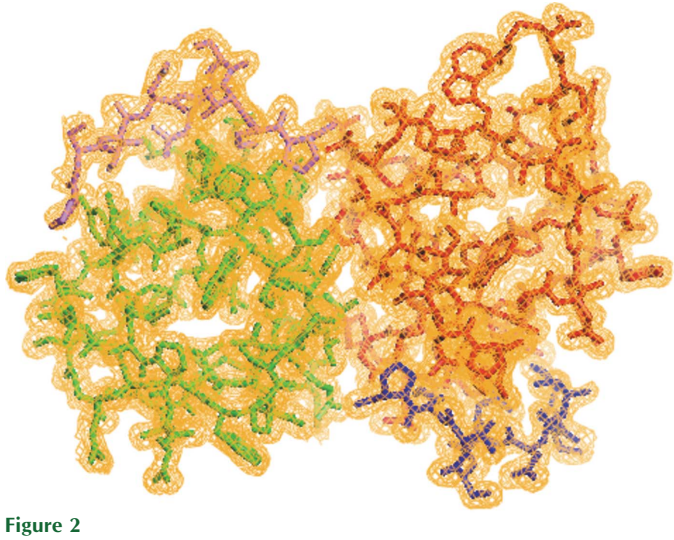


Figure 2
Electron-density map and model corresponding to the molecular-replacement solution for crystal wi13 after initial cycles of crystallographic refinement and manual model building. This solution was obtained by molecular replacement using the program *PHASER* v.1.3.1 (Storoni *et al.*, 2004) with the coordinates of the GADS-cSH3 monomer *A* (PDB code 1oeb) as a search model (Harkiolaki *et al.*, 2003), truncated of the first five amino-acid residues at the N-terminus and the last six amino-acid residues at the C-terminus. The SLP-76 peptide was not included in the original search model. The space group used for the molecular replacement was $P4_22_12$. The electron density is a $2F_o - F_c$ map as seen in the crystal asymmetric unit contoured at the 2.5σ level. The two GADS SH3 monomers are coloured red and green. The two SLP-76 peptides are coloured violet and blue. The figure was prepared using the program *PyMol* (http://pymol.sourceforge.net/).

with the calculated Matthews coefficient, the presence of two molecules of GADS-cSH3 each in complex with the SLP-76 peptide (Fig. 2). In addition, the mode of dimerization of the two GADS-cSH3 monomers in the crystal asymmetric unit (Fig. 2) reveals a previously undescribed dimerization mode for an SH3 domain. The refined coordinates of this structure will be used as a search model for solving the structures of all the other crystals obtained.

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